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Decorin, biglycan and their endocytosis receptor in rat renal cortex

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Background. Among the small proteoglycans, biglycan and decorin have been proposed to be potent modulators of TGF-β-mediated inflammatory kidney diseases. They were considered to become induced during glomerulonephritis and to subsequently inactivate the cytokine.

Methods. Decorin and biglycan as well as their endocytosis receptor were investigated in normal rat renal cortex, in anti-Thy-1 glomerulonephritis, in polycystic kidneys, in the remnant kidney following 5/6-nephrectomy, and in kidneys from the Milan normotensive strain by immunohistochemistry and *in situ* hybridization. Northern blots were used for the detection of mRNA expression for decorin and biglycan in isolated glomeruli. Functional aspects of the endocytosis of decorin and biglycan were studied in cultured mesangial cells.

Results. In the normal adult rat kidney decorin was expressed preferentially by Bowman's capsule and by interstitial connective tissue cells, but only in trace amounts by mesangial cells. In contrast, biglycan was found in tubular epithelial cells, in association with glomerular capillaries, podocytes and occasionally in the mesangium. In the tubulointerstitium of diseased kidneys (polycystic kidneys, 5/6-nephrectomy, kidneys from the Milan normotensive strain) there was a general up-regulation of decorin expression, while biglycan was localized only in distinct foci of fibrotic lesions. Glomerulosclerosis (5/6-nephrectomy, Milan normotensive strain) was associated with an increased staining for both decorin and biglycan within glomeruli. However, even in the anti-Thy-1 model of an acute mesangioproliferative glomerulonephritis where the greatest accumulation of decorin was found there was only a slight enhancement of decorin mRNA in isolated glomeruli. Decorin and biglycan become degraded upon receptor-mediated endocytosis. Immunohistochemical investigations indicated that the pattern of expression of the receptor protein correlated well with the immunolocalization of both decorin and biglycan. *In vitro* experiments with cultured mesangial cells provided direct evidence for the expression of the receptor and for the cell's capability to endocytose decorin as well as biglycan.

Conclusions. Decorin and biglycan are characterized by a distinct expression pattern in the normal rat kidney, whereas the presence of their endocytosis receptor protein correlates with the expression of both proteoglycans. Decorin is almost completely absent in the normal mesangium. Both proteoglycans become up-regulated in various models of renal disease. The mesangial accumulation of decorin in the anti-Thy-1 glomerulonephritis that is observed in spite of the only slightly enhanced mRNA expression could result from decreased decorin turnover and/or increased mesangial retention.

Key words: small proteoglycan endocytosis receptor, glomerulosclerosis, interstitial fibrosis.

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Decorin and biglycan are small chondroitin/dermatan sulfate proteoglycans that are both widely distributed in various extracellular matrices of mesenchymal origin. Biglycan can also be found around some epithelial cells [1]. According to the structure of their core proteins they are members of the family of proteoglycans with leucine-rich repeat structures [reviewed in 2–4], and they carry either a single glycosaminoglycan (GAG) chain in case of decorin or up to two chains for biglycan. Decorin is well known to interact via its core protein with several components of the extracellular matrix, especially with various collagens, and it may also have a role in growth control by influencing the expression of p21 [5]. The most noted feature of decorin knock out mice is skin fragility, most likely due to the presence of thick and irregularly shaped collagen fibrils [6].

The precise biological role of biglycan is still under discussion. It may associate with collagen fibrils, albeit with lower affinity than decorin [7], and it may interact with other matrix components, but its most common location is on the cell surface or in the pericellular space [1]. Both decorin and biglycan were shown to form complexes with transforming growth factor-beta (TGF-β), which in certain models results in the inactivation of the cytokine [8, 9] whereas in others its activity was either not influenced or even increased [10, 11]. In spite of these discrepancies it is obvious that the concentrations of biglycan and decorin in the tissues may be of great physiological importance.

The matrix content of both proteoglycans is logically the result of the rates of biosynthesis and degradation.

Whereas extracellular proteolytic degradation of both proteoglycans has been described, complete degradation requires the intralysosomal action of proteases, sulfatases and glycosidases, and hence endocytosis of the two macromolecules is a prerequisite for degradation. Fibroblasts and other cells of mesenchymal origin are known to efficiently internalize decorin by receptor-mediated endocytosis [12, 13]. Two proteins of 51 and 26 kD, respectively, which are present at the plasma membrane and in endosomes, bind decorin core protein with high affinity and are therefore considered as receptor proteins [12, 14]. It seems that biglycan core protein is recognized by the same receptor proteins as decorin [13, 15]. The receptor proteins also interact with membrane-associated heparan sulfate proteoglycans [14, 16]. The interaction of heparan sulfate with the receptor proteins leads to an inhibition of decorin endocytosis. Recently, the small proteoglycan endocytosis receptor has been isolated, and direct evidence for the internalization of the 51 kD receptor protein was provided [17].

Deficiencies of decorin and biglycan, both in the human and in experimental animals, showed major abnormalities in skin and bone [6, 18, 19]. The biological role of decorin and biglycan in the kidney has mainly been inferred from their TGF-β-binding and -neutralizing potency [8]. TGF-β is considered to be a key mediator of fibrosis in both experimental and human kidney diseases [20, 21]. On the basis of the observation of an apparently TGF-β-mediated dramatic up-regulation of biglycan and decorin in anti-Thy-1-initiated mesangioproliferative glomerulonephritis, it had been suggested that decorin is part of a negative feedback system controlling cell growth and matrix turnover [22]. Indeed, it could be shown that the administration of recombinant decorin [23] and the induction of decorin by gene transfer [24] successfully suppressed the proteinuria and the increased production of extracellular matrix components in the anti-Thy-1 model. On the other hand, immunocytochemical studies in normal rat kidney indicated the presence of decorin only in the adventitia of blood vessels. In experimental hydronephrosis decorin exhibited additionally a periglomerular and peritubular localization [25]. Such a localization would make it difficult to understand the role of locally produced decorin in TGF-β inactivation during glomerulonephritis.

Hence, the objectives of the present study were twofold: (*1*) to demonstrate the localization of decorin and biglycan in the normal rat kidney and in various models of renal disease, using a set of different antibodies; and (*2*) to provide evidence for the presence of the small proteoglycan endocytosis receptor in the normal and diseased rat kidney and to characterize this receptor in terms of the uptake of decorin and biglycan by cultured rat mesangial cells.

METHODS

Animals and isolation of glomeruli

Adult male Sprague-Dawley rats weighing 225 to 250 g (2 months of age) were supplied by Charles-River (Sulzfeld, Germany) and used for studies on the normal rat kidney. Fibrotic kidneys were taken from one-year-old male rats of the Milan normotensive strain (MNS) using age-matched Milan hypertensive animals as a control [26] and from 20-month-old male, heterozygous Han:SPRD rats (cy/+) suffering from polycystic kidney disease (PKD) [27]. Age-matched unaffected littermates (+/+) served as controls. Additionally, 5/6-nephrectomized, male Sprague-Dawley rats 15 weeks after renal ablation (5/6-Nx) or sham operation [28] were used. The anti-Thy-1 glomerulonephritis was induced by a single intravenous injection of mouse anti-rat Thy-1.1 IgG dissolved in PBS, clone OX-7 (BioTrend, Cologne, Germany), at a dose of 1 mg/kg body wt into 2-month-old, male Sprague-Dawley rats and kidneys were harvested at day 7. Age-matched control animals were receiving a single intravenous injection of PBS only. All surgical procedures were performed under anesthesia with hexobarbital (150 mg/kg body wt) and kidneys were perfused with cold 0.15 M NaCl before being removed. All animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Glomeruli were isolated by differential sieving according to the method of Spiro [29] and processed for Western blotting or RNA isolation.

Cell culture

Rat mesangial cells were kindly provided by Dr. E. Schulze-Lohoff, University of Erlangen, Germany, rat kidney fibroblasts were from ATCC (cat. No. 6509; Rockville, MD, USA) and rat skin fibroblasts were obtained from explant cultures. All cells were maintained in modified Eagle's minimum essential medium supplemented with 10% fetal calf serum, non-essential amino acids and antibiotics [12].

Immunohistochemistry

The following primary antibodies against decorin were applied in this study, their working dilution being given in brackets: rabbit α-rat PDS II (1:500), which was raised against rat decorin from rat fibroblast secretions [30]; rabbit LF 113 (1:300), being directed against a tetradecapeptide near the N-terminus of rat decorin core protein [31]; and LN1 (1:50), a monoclonal antibody against human decorin [32]. The specifity against decorin had been ascertained before by preabsorption and competition experiments. MAY-01 (1:500) is a newly developed chicken antibody against a dodecapeptide located near the N-terminus of the rat biglycan core protein (Ser67 - Gln78) that did not react at a 1:100 dilution with purified rat or human decorin; LF 106 (1:300) is a polyclonal rabbit antibody against a murine biglycan peptide of 15 amino acids (Arg12-Tyr26) [31]. The polyclonal rabbit antibody against rat small proteoglycan endocytosis receptor (1:100) as well as its affinity purified form (1:50 corresponding to

an approximately 1000-fold dilution of the original antiserum used for affinity purification) has been described recently [17].

For immunocytochemistry renal tissue was fixed with 4% formaldehyde in 18 mM sodium phosphate, pH 7.4/0.15 M NaCl (PBS). Paraffin sections (2 μm) were pretreated using a set of different enzymes: 0.05% bacterial protease type XXIV (EC 3.4.21.62; Sigma, Deisenhofen, Germany) for 2 to 15 minutes at 37°C, 0.05% protease type XIV from Streptomyces griseus (EC 3.4.24.31; Dakopats, Hamburg, Germany) for 2 to 10 minutes at room temperature, 7.5 mU/ml of chondroitin ABC lyase (EC 4.2.2.4; Sigma) for two hours at 37°C prior to the administration of antibodies raised against decorin or biglycan core protein [1] and with 25 mU/ml of heparitinase (EC 4.2.2.8; Seikagaku Kogyo, Tokyo, Japan) for two hours at 37°C in order to digest the heparan sulfate prior to incubation with the antibody against the small proteoglycan endocytosis receptor. The sections were subsequently blocked overnight at 4°C using 10% normal goat serum (Dakopats) and 5% bovine serum albumin (BSA; Sigma) in 0.05 M Tris-buffered saline (TBS) and incubated with the primary antibody in a moist chamber for one hour at ambient temperature. After washing, the sections were incubated with secondary antibodies (Dakopats): mouse anti-rabbit immunoglobulins (1:125 in TBS containing 1% BSA for 30 min at room temperature) and subsequently with rabbit anti-mouse (1:30 in TBS containing 1% BSA and 12.5% normal rat serum) for 30 minutes at room temperature. In the case of the monoclonal antibody LN1 the first bridging antibody was omitted. For MAY-01 rabbit anti-chicken immunoglobulins (Sigma; 1:100 in TBS containing 1% BSA) were used as the first bridging antibodies. To complete the sandwich technique a soluble complex of alkaline phosphatase anti-alkaline phosphatase (APAAP; Dakopats) was added [33]. The last two steps were repeated to enhance the intensity of the reaction. The enzyme label was visualized with naphthol AS-MX-phosphatase (Sigma) and fast red dye (Sigma) in the presence of levamisole (Sigma) in order to block endogenous alkaline phosphatase. The slides were counterstained with Mayer's hemalaun (Sigma) and covered with glycerol-gelatin. The specifity of immunolabeling was tested by omitting the primary antibody and by using non-immune serum or "unspecific" IgG instead. In the case of the endocytosis receptor, the specificity was tested additionally by using an antiserum in which the receptor-specific antibodies had been removed by adsorption to immobilized receptor protein.

Northern blot analysis

Northern blot analysis was performed as described previously [34]. Briefly, RNA was extracted from glomeruli or mesangial cells using TRI-zol (Life Technologies, Eggenstein, Germany). All probes were radiolabeled with [^{32}P]dCTP (Amersham-Buchler, Braunschweig, Germany) using the DECAprime DNA labeling kit (Ambion, Austin, TX, USA). Hybridization was carried out overnight at 42°C. The filters were exposed to Kodak X-OMAT AR film at −80°C for one day.

The cDNA probes for rat biglycan and decorin were obtained by RT-PCR. Total rat kidney RNA (1 μg) was reverse-transcribed with Super Script II reverse transcriptase (Life Technologies) and random hexanucleotides for cDNA priming. Double-stranded cDNAs were generated by amplification of the reverse-transcribed RNA by using primers that contained additionally appropriate sequences with restriction sites for *Eco*RI and *Not*I, respectively. For decorin the forward primer was 5′-TGC TGG AAT TCC CCG GAT TAA AAG GTG GTG A-3′, and the reverse and complement primer was 5′-CTC GAG CGG CCG CTG AGG CTG TTT GGG AGT TAC-3′, thus yielding a rat decorin cDNA encompassing bp 179-1238 [35]. For rat biglycan the respective primers were 5′-TGC TGG AAT TCC CTC CCC AGG AAC ATT GAC-3′ and 5′-CTC GAG CGG CCG CGC CAT GGT GGC TAC CAC TG-3′, which allowed the generation of a biglycan cDNA between bp 123 and 1230 [36]. After restriction endonuclease treatment the PCR products were cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA) and verified by sequencing.

In situ hybridization

A decorin cDNA probe comprised nucleotides 89 to 984 of the rat decorin cDNA [35].

A biglycan cDNA probe was obtained from a full-length human biglycan cDNA [37] being cloned into the pBluescript plasmid (Stratagene, La Jolla, CA, USA). After digestion with *Eco*RI/*Hin*cII a 831 bp fragment was cloned into pGEM-4Z (Promega, Madison, WI, USA) and subsequently subjected to restriction with *Stu*I/*Hin*cII, thus yielding a 278 bp biglycan insert (position 240-518) with a homology to rat biglycan of 88.4%. Sense and antisense riboprobes were transcribed *in vitro* from the linearized plasmids using digoxygenin-labeled UTP and the SP6/T7-RNA polymerase kit (DIG RNA Labeling Kit SP6/T7; Boehringer Mannheim, Germany).

Tissue sections were deparaffinized with xylene and rehydrated in a descending ethanol series. Cell membranes were permeabilized by digestion for three to six minutes at 37°C with 3 μg/ml proteinase K (Boehringer) in 20 mM Tris/HCl, pH 8.0, containing 2 mM $CaCl_2$. After refixation with 4% paraformaldehyde in PBS for 20 minutes on ice and acetylation with 0.1 M triethanolamine/0.25% acetic anhydride for 10 minutes the specimens were incubated with prehybridization buffer (50% deionized formamide, 1 × Denhardt's solution, 0.5 mg/ml denatured salmon sperm DNA, 0.25 mg/ml yeast tRNA, 10% dextran sulfate and 1% 1 M DTT in 2 × SSC) in a humid chamber at 49°C for two hours. The hybridization solution contained the appropriate riboprobe at a final concentration of about 7.5 ng/ml in prehybridization buffer. Hybridization was for 16

hours at 49°C. Thereafter, the specimens were washed twice for 20 minutes with 50% formamide and 1% 2-mercaptoethanol in 2 × SSC, 20 minutes in 2 × SSC, and 10 minutes in 0.1 × SSC at 50°C. Immunological detection of the probe/mRNA hybrids was performed as described [38] with some modifications. The sections were blocked with 2% normal sheep serum and 0.05% Triton X-100 in 2 × SSC for two hours, washed twice for five minutes with 50 mM Tris/HCl, pH 7.5, containing 225 mM NaCl (buffer 1) before the antibody solution (alkaline phosphatase-conjugated Fab fragments of anti-digoxygenin antibodies (Boehringer Mannheim) at a dilution of 1:1000, 1% normal sheep serum, 0.3% Triton X-100 in buffer 1) was applied in a humid chamber for two hours at 37°C. To remove the unbound antibody conjugate, the slides were washed twice with buffer 1. Thereafter, they were equilibrated for 10 minutes with 100 mM Tris/HCl, pH 9.5, containing 100 mM NaCl and 50 mM $MgCl_2$ (buffer 2). Endogenous alkaline phosphatase was blocked by 5 mM levamisole, and the digoxygenin/anti-digoxygenin conjugates were visualized upon reaction with 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate/0.3 mg/ml nitroblue tetrazolium (FAST BCIP/NBT; Sigma) in 100 mM Tris/HCl, pH 9.5/5 mM $MgCl_2$ for 16 hours at 4°C. The slides were then dehydrated and observed without counterstain.

Other methods

Endocytosis experiments were performed exactly as described before [12]. [^{35}S]sulfate-labeled decorin was either obtained from the media of human skin fibroblasts or from the media of 293 cells stably transfected with a human decorin cDNA [39]. [^{35}S]sulfate-labeled biglycan was analogously obtained from 293 cells transfected with a human biglycan cDNA. Proteoglycan purification was achieved by ion exchange chromatography [12] followed by chromatography on a 1 ml phenyl-Sepharose column prepared in a Pasteur pipet and equilibrated with 2 M $(NH_4)_2SO_4$ in 10 mM potassium phosphate, pH 6.8. The column was washed with 0.2 M $(NH_4)_2SO_4$ in potassium phosphate buffer, and small proteoglycans were subsequently eluted with water. Since endocytosis of decorin and biglycan is followed by intralysosomal degradation, and the resulting products are in part released into the culture medium, endocytosis is represented by the sum of the intracellular radioactivity and the ethanol-soluble radioactivity in the culture medium. For comparison, endocytosis is usually expressed as clearence rate, giving the volume of incubation medium (in microliters) cleared from the labeled material per hour and per milligram cell protein. Western blotting using polyclonal antibodies against the rat decorin endocytosis receptor was performed as described earlier [13].

RESULTS

Immunolocalization and mRNA expression of decorin and biglycan in normal rat kidney

In the adult rat kidney decorin was immunolocalized in the interstitial connective tissue, with focal concentrations around tubular structures, in the adventitia of blood vessels and in the perivascular space, and in the Bowman's capsule of glomeruli. Only trace amounts of decorin could be detected in the mesangium after employing two successive rounds of the APAAP technique (Fig. 1). These staining patterns were observed with a polyclonal antibody against the full-length core protein prepared from rat fibroblast cultures (α-PDS) (Fig. 1), a polyclonal antiserum against a decorin-derived peptide linked with horseshoe crab hemocyanin (LF113) and a monoclonal antibody against human decorin that cross-reacts with the proteoglycan from rat tissue (LN1). With all three antibodies, however, it was necessary to pretreat the tissue sections with one of the proteases or with chondroitin ABC lyase, but the different protocols described for proteolytic pretreatment yielded similar results.

In agreement with the histochemical findings, *in situ* hybridization for decorin gave a distinct signal in Bowman's capsule cells and a stronger one in the adventitia and around tubules, that is, presumably in mesenchymal cells located between tubuli. In the mesangium the signal was confined to few mesangial cells (Fig. 1).

Collecting ducts, distal tubules as well as vessel walls stained strongly positive for biglycan. It could also be detected within glomeruli where it was mainly associated with capillaries, but was visible within the mesangium and around podocytes, too (antibody MAY-01; Fig. 1). An identical staining pattern for biglycan in the normal rat kidney could be observed with the LF 106 antiserum (data not shown). *In situ* hybridization studies supported the cytochemical results (Fig. 1).

As expected from the relatively strong signals seen in Bowman's capsule cells upon *in situ* hybridization, Northern blot analyses of total RNA from isolated rat glomeruli demonstrated the presence of both decorin and biglycan mRNA (Fig. 2A).

Presence of the small proteoglycan endocytosis receptor in normal rat renal cortex

The quantity of decorin and biglycan in the tissue represents the balance between biosynthesis and secretion on the one hand and endocytosis and degradation on the other hand. Since we had recently been able to raise a monospecific antiserum against the rat decorin endocytosis receptor, which is probably also responsible for the uptake of biglycan, we searched for the presence of the receptor in the kidney. As it is shown in Figure 3, the endocytosis

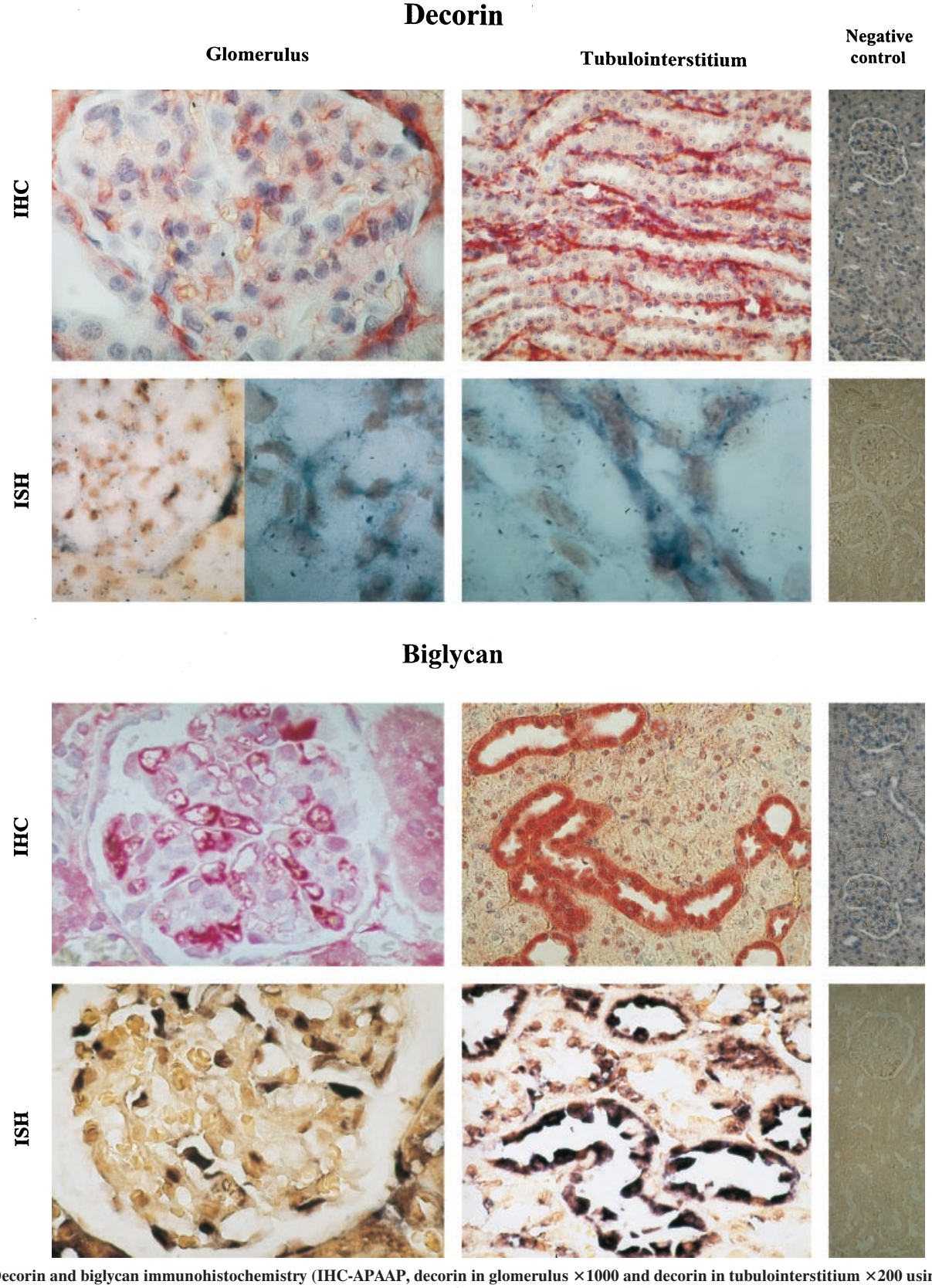


Fig. 1. Decorin and biglycan immunohistochemistry (IHC-APAAP, decorin in glomerulus ×1000 and decorin in tubulointerstitium ×200 using α-PDS 1:500 after pretreatment with 0.05% protease type XXIV for 10 min at 37°C; biglycan in glomerulus ×600, biglycan in tubulointerstitium ×400 using MAY-01 1:500 after pretreatment with 7.5 mU/ml of chondroitin ABC lyase for 2 hours at 37°C) and *in situ* hybridization (ISH, decorin in glomerulus: left panel ×600, right panel ×1200 and in tubulointerstitium ×1200; biglycan in glomerulus ×1000 and in tubulointerstitium ×600), using digoxygenin-labeled anti-sense riboprobes, in the glomerulus and tubulointerstitium of normal rat kidney. Negative controls for IHC were performed using non-immune rabbit IgG (for decorin) and non-immune chicken IgG (for biglycan) as substitution for the primary antiserum (×100). As negative controls for ISH digoxygenin-labeled sense riboprobes for decorin and biglycan were used (×100).

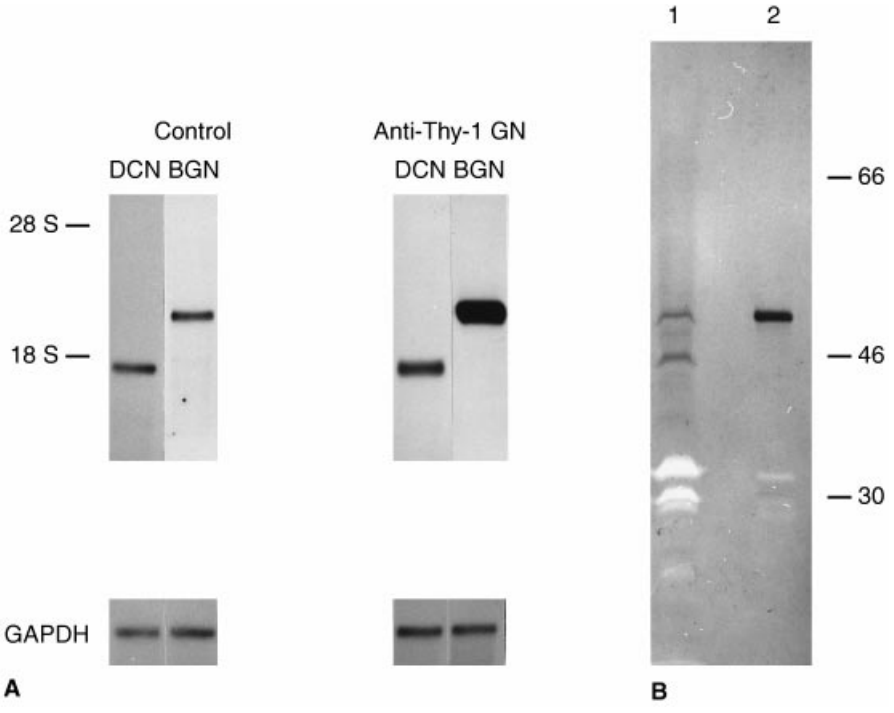


Fig. 2. (*A*) Northern blot analysis of decorin (DCN 1.8 kb) and biglycan (BGN 2.6 kb) in glomeruli from control animals and from the anti-Thy-1 glomerulonephritis (anti-Thy-1 GN). 28S and 18S rRNA correspond to 4.8 and 1.9 kb, respectively. Densitometric analysis was normalized to GAPDH expression (lower panel). (*B*) Western blot analysis of the small proteoglycan endocytosis receptor in glomeruli from normal kidneys showing 51 kD and 46 kD protein bands (lane 1) and purified receptor protein (51 kD) from rat brain (lane 2).

receptor exhibited a similar distribution as both decorin and biglycan.

The observed staining, however, depended on the type of digestion used prior to the administration of the first antibodies. "Mild" digestion using protease type XIV for 2 to 10 minutes at room temperature or protease type XXIV for up to eight minutes at 37°C revealed traces of staining in the glomerulus and strong positivity in distal tubules and collecting ducts (Fig. 3A). Digestion with heparitinase showed additional staining in peritubular mesenchymal cells (data not shown). "Strong" digestion with protease XXIV over 10 minutes at 37°C almost completely abolished this pattern of staining and revealed the localization of the receptor in Bowman's capsule and in mesenchymal cells around tubules (Fig. 3B).

Western blots using polyclonal antibodies to the small proteoglycan endocytosis receptor detected the presence of a 51 kD protein in the homogenate of isolated glomeruli (Fig. 2B, lane 1) in agreement with the purified endocytosis receptor protein from rat brain (Fig. 2B, lane 2). An additional protein band of 46 kD could be detected in isolated glomeruli, which corresponds well with earlier findings [17] and most likely represents a degradation product of the receptor because of its cross-reactivity with affinity-purified antibodies.

Immunolocalization of decorin, biglycan and their endocytosis receptor in fibrotic rat kidneys

In light of the potential role of small proteoglycans in the development of renal fibrosis, we studied the expression of decorin, biglycan and of their endocytosis receptor in different models of tubulointerstitial fibrosis and glomerulosclerosis. The Han:SPRD rat represents a model of polycystic kidney disease with an accumulation of a collagenous matrix around atrophic and dilated tubules without any glomerulosclerosis. In the remnant kidney after renal ablation (5/6-Nx) glomeruli become hypertrophic and sclerotic, and considerable quantities of extracellular matrix accumulate in the tubulointerstitium. In the Milan normotensive strain (MNS) focal glomerulosclerosis and interstitial fibrosis can be observed. In all these models of experimental kidney disease immunohistochemistry revealed a remarkable over-expression of decorin in the tubulointerstitium compared to the normal rat kidney. In 5/6-Nx and

Fig. 3. Immunostaining of small proteoglycan endocytosis receptor in normal rat kidney cortex. (*A*) After pretreatment with protease type XIV for six minutes at room temperature demonstrating traces of staining in the glomerulus and strong positivity in distal tubules and collecting ducts (APAAP, glomerulus ×1000, tubulointerstitium ×400). (*B*) After pretreatment with protease type XXIV for 12 minutes at 37°C showing weak positivity in Bowman's capsule and in peritubular mesenchymal cells (APAAP, glomerulus ×600, tubulointerstitium ×1000). Negative controls were performed using an antiserum from which the receptor-specific antibodies had been removed by adsorption to immobilized receptor protein (×100).

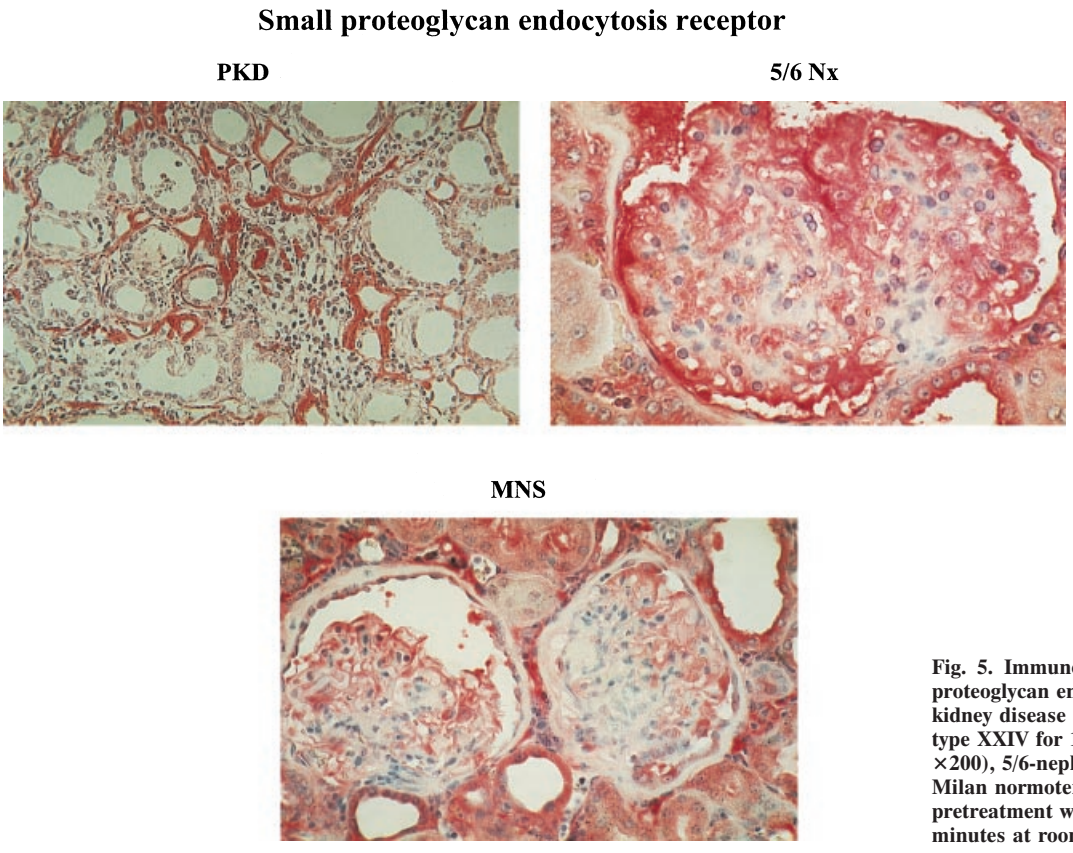
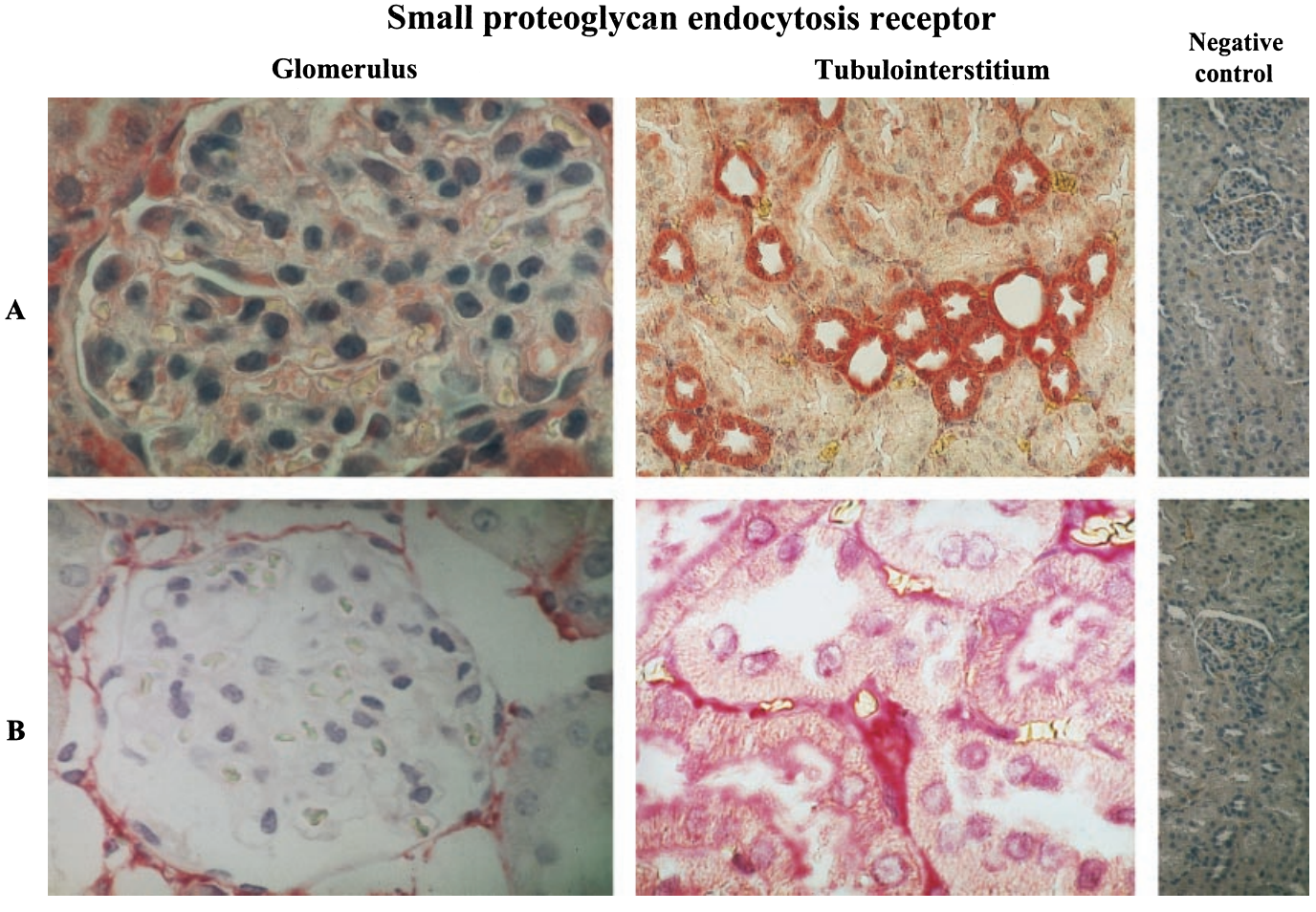


Fig. 5. Immunohistochemistry of small proteoglycan endocytosis receptor in polycystic kidney disease after pretreatment with protease type XXIV for 12 minutes at 37°C (PKD, ×200), 5/6-nephrectomy (5/6-Nx, ×600) and Milan normotensive strain (MNS, ×320) after pretreatment with protease type XIV for six minutes at room temperature (APAAP).



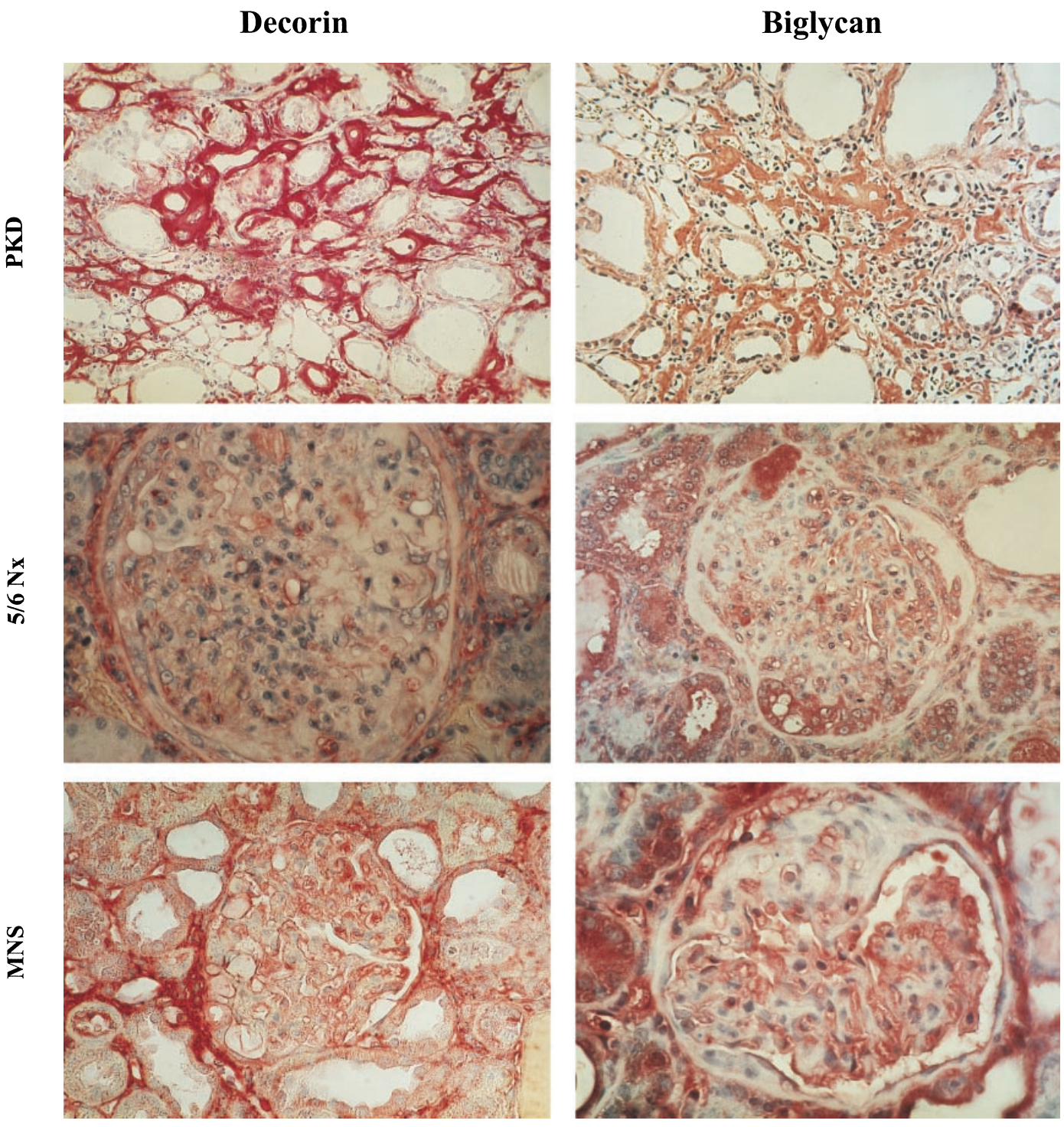


Fig. 4. Immunohistochemistry of decorin (α-PDS, 1:500 after pretreatment with 0.05% of protease type XXIV for 10 min at 37°C) and biglycan (MAY-01, 1:500 after pretreatment with 7.5 mU/ml of chondroitin ABC lyase for 2 hr at 37°C) in polycystic kidney disease (PKD, ×200), 5/6-nephrectomy (5/6-Nx,decorin ×600, biglycan ×400) and Milan normotensive strain (MNS, decorin ×400, biglycan ×600) (APAAP).

MNS an increased immunostaining for decorin was also seen within afflicted glomeruli (Fig. 4).

In all of these models the quantity of immunoreactive biglycan appeared to be slightly increased in the tubulointerstitial space, while the staining in distal tubules and collecting ducts was comparable with the normal kidney. Biglycan staining was more pronounced in polycystic kidney disease with additional labeling of atrophic proximal

tubules. In the model of experimental glomerulosclerosis (5/6-Nx, MNS) there was clear overexpression of biglycan in the mesangium (Fig. 4). Immunolocalization of either proteoglycan in the respective age-matched control kidneys was identical with the normal kidney (data not shown).

The small proteoglycan endocytosis receptor was also stained more intensely in fibrotic kidneys as compared to the normal parenchyma, and it co-localized quite well with areas staining for both decorin and biglycan (Fig. 5). Again, "mild" and "strong" digestion gave different staining patterns. With "mild" digestion relatively strong staining for the receptor was visible in distal tubules and collecting ducts (data not shown) and within the mesangium of sclerotic glomeruli (Fig. 5). "Strong" digestion resulted in marked immunolabeling of peritubular mesenchymal cells in fibrotic lesions, particularly in the model of polycystic kidney disease (Fig. 5).

Expression and immunolocalization of decorin, biglycan and their endocytosis receptor in acute mesangioproliferative glomerulonephritis

A single injection of the monoclonal anti-thymocyte antibody (anti-Thy-1.1) in the rat leads to mesangiolysis followed by mesangial proliferation and accumulation of extracellular matrix. Strong focal immunostaining of both decorin and biglycan could be observed in the mesangium of injured glomeruli (Fig. 6). Parallel to the focal accumulation of decorin and biglycan, the endocytosis receptor was more strongly stained in afflicted glomeruli, co-localizing with areas of matrix accumulation (Fig. 6). Isolated glomeruli from anti-Thy-1 kidneys, harvested at day 7, revealed only a slight enhancement of decorin mRNA expression (1.5 ± 0.2 times, $N = 3$) compared to glomeruli from control kidneys, while biglycan mRNA expression was increased 6.2 ± 0.4 times ($N = 3$; Fig. 2A). *In situ* hybridization studies corroborated the finding of an increased biglycan expression, while the signal for decorin was at the limit of detection (Fig. 6).

Endocytosis of decorin and biglycan by cultured mesangial cells

To gain further insight into the dynamics of small proteoglycan metabolism rat mesangial cells were cultured *in vitro*, and their capability to endocytose small proteoglycans was investigated. Although these cells may dedifferentiate *in vitro*, tissue culture systems nevertheless may indicate metabolic capabilities of defined cell types. In a first set of experiments decorin uptake by rat skin fibroblasts, renal fibroblasts and mesangial cells was compared. [^{35}S]sulfate-labeled decorin was prepared under non-denaturing conditions from the conditioned media of human skin fibroblasts and added at a concentration of 40,000 cpm/ml culture medium to the three different rat cell types. After four hours of incubation uptake was measured and expressed as clearance rate (volume of culture medium cleared from radioactive decorin per hour and mg cell protein). The clearance rates were 37 μl/hr and mg for skin fibroblasts, 34 μl/hr and mg for renal fibroblasts, and 27 μl/hr and mg for mesangial cells. Since fluid-phase uptake accounts for less than 0.1 μl/hr and mg, these data indicate the capacity of mesangial cells for receptor-mediated uptake of decorin.

For a comparative study of the endocytosis of decorin and biglycan the respective [^{35}S]sulfate-labeled proteoglycans were obtained from the media of 293 cells that had been stably transfected with human biglycan and decorin cDNA, respectively. Non-transfected 293 cells neither synthesize decorin nor biglycan, and the two proteoglycans could therefore be separately obtained by a purification scheme avoiding denaturing agents. When cells were incubated in the presence of labeled proteoglycan (100,000 to 200,000 cpm/ml) for three hours, clearance rates of 16.2 ± 3.2 μl/hr and mg ($N = 9$) and 49.9 ± 8.7 μl/hr and mg ($N = 6$) were obtained in several independent experiments for the uptake of decorin and biglycan, respectively. A typical experiment for the dose-dependency of uptake is shown in Figure 7. The data show that a greater proportion of radioactive biglycan than of radioactive decorin could be endocytosed. This finding, however, should be judged in the context that, in contrast to decorin, the majority of biglycan molecules is linked with two glycosaminoglycan chains and therefore may have more ^{35}S-label per molecule. It is not yet understood why there was a greater difference between mesangial cells and fibroblasts in the endocytosis of biglycan than in the uptake of decorin. The data available so far are in favor of assuming a single receptor for both proteoglycans.

DISCUSSION

Small proteoglycans, especially decorin, are intensely studied components of the extracellular matrix of the kidney because of their suggested capability to control mesangial proliferation and matrix accumulation. However, somewhat conflicting data on their presence in the kidney have been reported. By using a monoclonal antibody against decorin core protein Diamond et al had shown only periadventitial immunolabeling of decorin and no glomerular reactivity in the normal rat kidney [25]. However, it could not be excluded that the reactive epitope was masked at other locations, for example, by the glycosaminoglycan chain [40]. We have therefore employed, using a very sensitive staining technique, two polyclonal antisera in addition to the monoclonal antibody used before [25]. The three reagents gave identical staining patterns. However, pretreatment with either proteases or chondroitin ABC lyase was necessary. From the results obtained, it seems safe to conclude that decorin is present in the normal

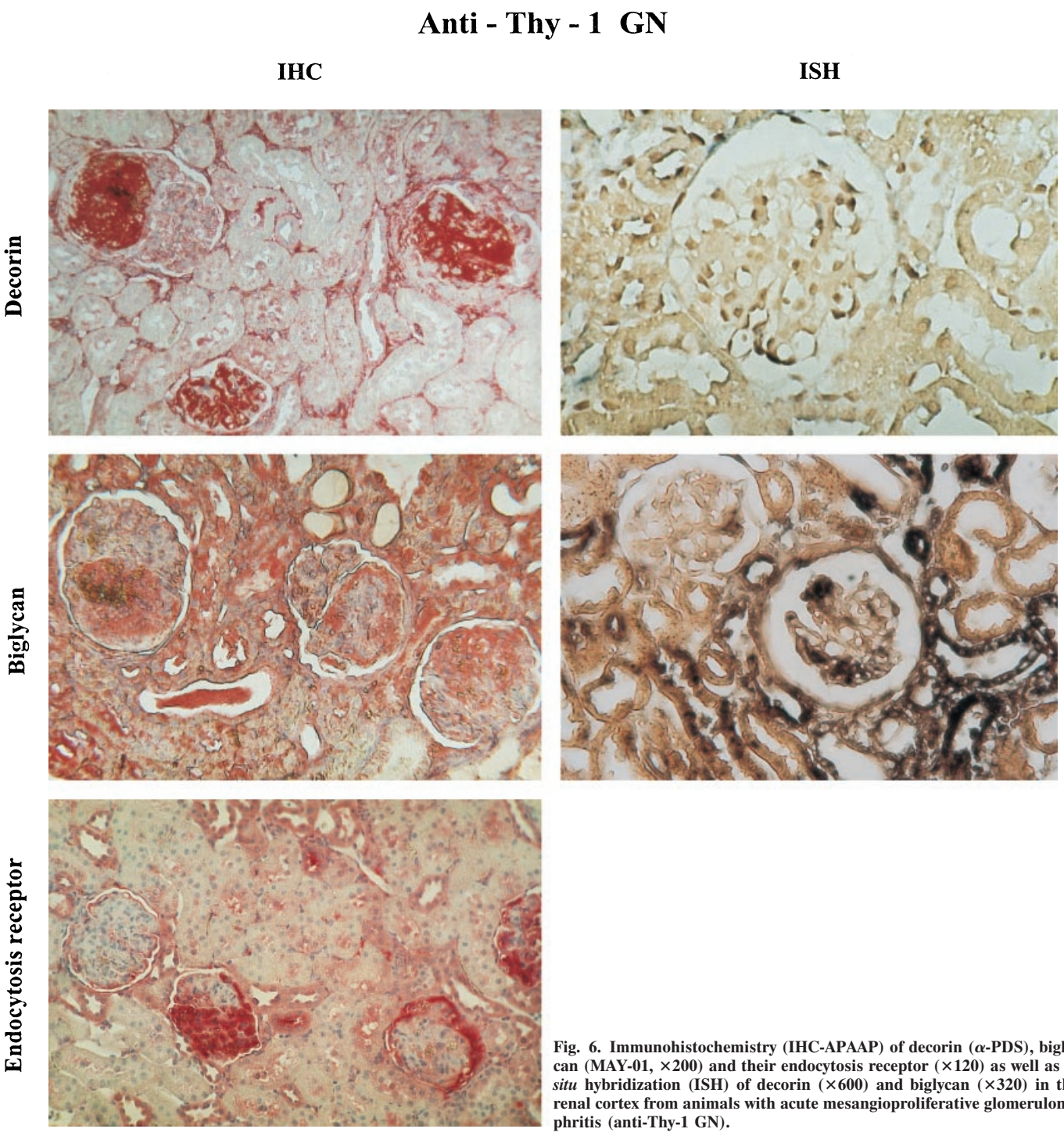


Fig. 6. Immunohistochemistry (IHC-APAAP) of decorin (α-PDS), biglycan (MAY-01, ×200) and their endocytosis receptor (×120) as well as *in situ* hybridization (ISH) of decorin (×600) and biglycan (×320) in the renal cortex from animals with acute mesangioproliferative glomerulonephritis (anti-Thy-1 GN).

glomerulus preferentially in Bowman's capsule and not in the mesangium. The demonstration of decorin in cultured glomeruli [22] and of decorin mRNA in freshly isolated glomeruli [41, this study] can, therefore, not be attributed solely to the presence of mesangial cells.

The presence of biglycan in the glomeruli was to be expected since biglycan seems to be constitutively expressed by endothelial cells [42]. It is also present in the mesangium and podocytes, but the focal accumulation in some glomeruli remains unexplained primarily because of

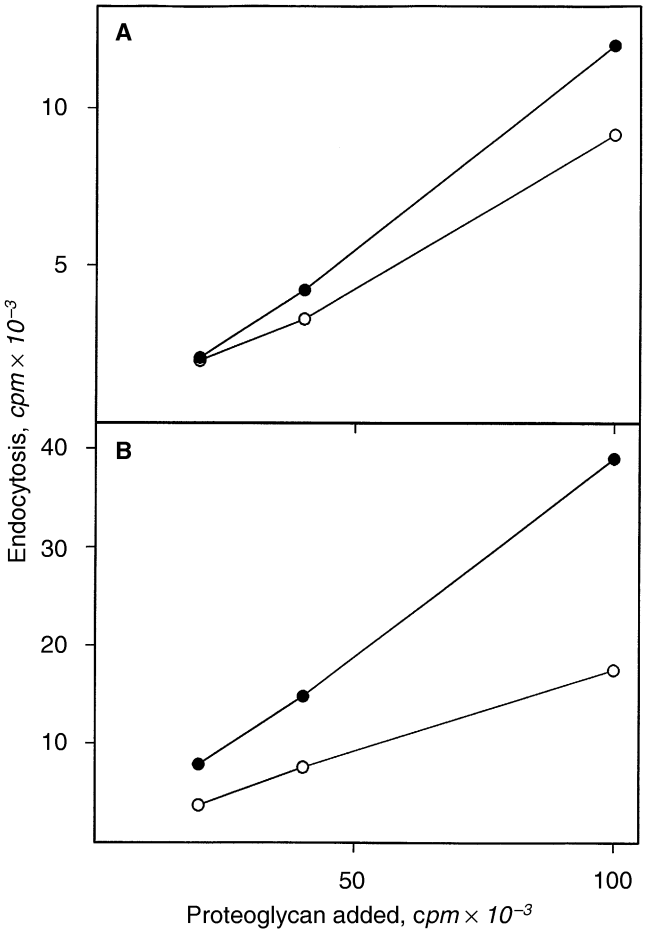


Fig. 7. Endocytosis of decorin (*A*) and biglycan (*B*) by human skin fibroblasts (●) and rat mesangial cells (○)**.** Cells were incubated for three hours with the indicated amounts of [^{35}S]sulfate-labeled recombinant human proteoglycan expressed in 293 cells.

the insufficient knowledge of potential biglycan-binding proteins. The association of biglycan with the epithelia of collecting ducts had been mentioned before [1] whereas the presence in distal tubules is described here for the first time, to our knowledge.

As the quantity of the small proteoglycans results from the net rates of biosynthesis and degradation that occurs after receptor-mediated endocytosis, we have also investigated the immunocytochemical localization of the endocytosis receptor. From competition and direct binding experiments it is likely that the 51 kD decorin receptor protein is also involved in the uptake of biglycan [13, 15]. The present study, using affinity-purified antibodies and the crude antiserum with similar results, clearly demonstrates the presence of the receptor, albeit in small amounts, in mesangial cells as well as in cells of the Bowman's capsule. In addition, immunostaining was also seen in distal tubules, collecting ducts and interstitial mesenchymal cells. This pattern of expression of the receptor protein correlates well with the immunolocalization of both decorin and biglycan. It is also in agreement with our *in vitro* results showing that both mesangial cells and renal fibroblasts in culture are capable of receptor-mediated uptake of decorin and biglycan. It should be kept in mind, however, that reactive epitopes of the receptor protein may be masked to a different degree depending on the localization within the tissue. From a functional point of view it is also noteworthy that the extent of small proteoglycan endocytosis is not only a function of their concentration and of the receptor expression but additionally depends on the quantity and domain structure of cell-associated heparan sulfate [16]. Studies are underway to address these questions in isolated glomeruli and in cultured mesangial cells.

In the models of experimental renal disease studied here, an increased immunostaining for both decorin and biglycan within glomerular lesions was observed (5/6-Nx, MNS). A more intense staining for decorin was seen throughout the tubulointerstitium whereas biglycan accumulated in distinct foci (5/6-Nx, MNS and PKD). For decorin, similar findings were obtained in kidney biopsies from patients with renal diseases of various etiologies [43, 44]. An increased decorin staining was found in the tubulointerstitial space, and the proteoglycan turned out to be a good predictor of the severity of interstital fibrosis and the progression of renal failure [43].

In the present investigation the strongest overexpression of decorin and biglycan within the mesangium was detected in acute mesangioproliferative glomerulonephritis (anti-Thy-1 model); however, this finding was limited to certain glomeruli and the average decorin mRNA level of isolated glomeruli rose only by a factor of 1.5. In the anti-Thy-1 model of glomerulonephritis the role of TGF-β for the development of glomerulosclerosis and the beneficial effect of decorin administration have been clearly demonstrated [23]. In other models of glomerulosclerosis, however, the increase of decorin appeared to be greater in the tubulointerstitium than in the mesangium. This increase was seen in spite of the apparent up-regulation of the endocytosis receptor. With regard to the potential regulatory role of decorin during development of TGF-β-mediated renal fibrosis our results, are in agreement with the assumption that the up-regulation of decorin allows the formation of a ternary complex of matrix-bound decorin and TGF-β to a greater extent, thereby withdrawing the cytokine from its cell membrane receptors. Considering the rather modest increase of decorin, at least in the glomeruli, it also seems possible that low levels of soluble decorin bind to low levels of soluble TGF-β, and that these complexes are not retained at the site of formation but are removed by diffusion and blood flow or, alternatively, by endocytosis. Thus, the levels of decorin in tissue sections do not necessarily reflect the effects of the proteoglycan on the bioactivity of TGF-β, and it may be worthwhile to follow blood levels of decorin during the development of tissue fibrosis.

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